Narrow Band Ultraviolet B Treatment for Human Vitiligo Is Associated with Proliferation, Migration, and Differentiation of Melanocyte Precursors

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In vitiligo, the autoimmune destruction of epidermal melanocytes produces white spots that can be repigmented by melanocyte precursors from the hair follicles, following stimulation with UV light. We examined by immunofluorescence the distribution of melanocyte markers (C-KIT, DCT, PAX3, and TYR) coupled with markers of proliferation (KI-67) and migration (MCAM) in precursors and mature melanocytes from the hair follicle and the epidermis of untreated and narrow band UVB (NBUVB)-treated human vitiligo skin. NBUVB was associated with a significant increase in the number of melanocytes in the infundibulum and with restoration of the normal melanocyte population in the epidermis, which was lacking in the untreated vitiligo. We identified several precursor populations (melanocyte stem cells, melanoblasts, and other immature phenotypes), and progressively differentiating melanocytes, some with putative migratory and/or proliferative abilities. The primary melanocyte germ was present in the untreated and treated hair follicle bulge, whereas a possible secondary melanocyte germ composed of C-KIT+ melanocytes was found in the infundibulum and interfollicular epidermis of UV-treated vitiligo. This is an exceptional model for studying the mobilization of melanocyte stem cells in human skin. Improved understanding of this process is essential for designing better treatments for vitiligo, ultimately based on melanocyte stem cell activation and mobilization.

Journal of Investigative Dermatology (2015) **135,** 2068–2076; doi:10.1038/jid.2015.126; published online 14 May 2015

INTRODUCTION

Vitiligo is the most common acquired type of leukoderma, causing significant social and psychological difficulties in all patients. The hallmark of the disease is white patches of the skin, the result of epidermal melanocyte destruction (Picardo and Taïeb, 2010; Birlea *et al.*, 2012). Melanocyte loss in vitiligo is caused by immune attack operated by melanocyte-specific T cells, innate immunity, and melanocyte-specific antibodies (Kemp *et al.*, 2011; Mosenson *et al.*, 2013). Reversing vitiligo depigmentation is a classic example of regenerative medicine, requiring activation of hair follicle and epidermal melanocyte precursors by UV light and/or drugs (Birlea *et al.*, 2013). Experimental observations in human vitiligo (Ortonne *et al.*, 1979, 1980; Cui *et al.*, 1991) and repigmentation transgenic mice models (Nishimura *et al.*, 2002; Chou *et al.*, 2013; Yamada *et al.*, 2013) have shown that, during UV exposure, melanocyte precursors proliferate and migrate along the infundibulum outer root sheath to the interfollicular epidermis (further referred to as IE or the epidermis) and repopulate the epidermal areas devoid of melanocytes. Yet, the cell populations that emerge during vitiligo treatment and the actual sequence of repigmentation (proliferation/migration/differentiation) in human vitiligo have not been described. Despite the progress made in the genetics of depigmentation (Spritz, 2013), vitiligo repigmentation in humans remains an enigma, first due to the impossibility of following in real time the melanocyte migration to the human epidermis, and second because the available mouse models and human skin equivalents cannot accurately reproduce the repigmentation process in patients undergoing phototherapy.

To better understand the human repigmentation pathways, we developed a model using biopsies of untreated and narrow band UVB (NBUVB)-treated human vitiligo skin in combination with fluorescent immunostaining to define and

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Abbreviations: DCT, dopachrome-tautomerase; MCAM, melanoma cell adhesion molecule; NBUVB, narrow band UVB; qRT-PCR, quantitative real-time reverse-transcriptase–PCR; TYR, tyrosinase

Received 8 December 2014; revised 11 March 2015; accepted 16 March 2015; accepted article preview online 30 March 2015; published online 14 May 2015

analyze the distribution of melanocyte precursors and differentiated melanocytes in the epidermis and hair follicles. To confirm some of the immunostaining results, we evaluated the expression of four pigmentation genes activated during UV exposure [*PAX3*, *tyrosinase* (*TYR*), *dopachrome-tautomerase* (*DCT*), and *KIT*]. We show that NBUVB treatment was associated with increased number of proliferative, putatively migratory, and differentiating populations of melanocytes in the infundibulum and the epidermis, supported by a primary melanocyte germ in the bulge and infundibulum and a possible secondary germ in the infundibulum and the epidermis. We also show that long-term intermittent NBUVB exposure was associated with high activation of pigmentation genes in the epidermis, as compared with the bulge.

RESULTS

Mapping the key regions of hair follicles

The first part of our study was focused on four regions: the epidermis, hair follicle infundibulum, hair follicle bulge, and hair follicle bulb (Figure 1). We divided the hair follicle into four portions from distal to proximal ends: infundibulum [between the epidermal ostium superiorly and the sebaceous gland duct inferiorly; Figure 1a], bulge (from the insertion of sebaceous gland duct above and arrector pili muscle below (Figure 1b and c)), sub-bulge (proximal to the bulge and distal to the bulb), and bulb—the base of hair follicle (Figure 1d). The stem cell population in the bulge was CD200+/K15+; the arrector pili muscle was desmin+ (Figure 1c). Both CD200 and K15 antibodies were strongly and differentially expressed in the outer root sheath in cells that adhered to the basement membrane. Infundibulum outer root sheath cells exhibited a CD200 − /K15 − phenotype (Figure 1a).

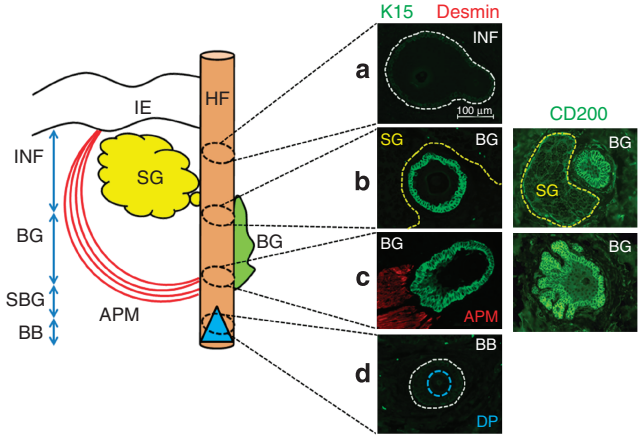

Figure 1. Mapping the anatomic regions of the hair follicle (HF). Anti-human K15 (green; labels epithelial stem cells) was paired with anti-human desmin (red; labels the arrector pili muscle (APM)); anti-human CD200 (green; labels epithelial stem cells) was immunostained by itself. HF infundibulum (INF) (**a**) was the region K15 − /CD200 − located between interfollicular epidermis (IE) and insertion of sebaceous gland (SG). Bulge (BG) (**b** and **c**) was the region K15+/CD200+ delineated by the SG duct superiorly and by the insertion of the APM inferiorly. The sub-BG (SBG) was located proximal to the bulge and distal to the bulb (BB). The bulb (**d**) was the region K15 − /CD200 − located at the base of HF, in which the dermal papilla (DP; delineated with blue line) was visible internally.

Immunohistochemical localization of melanocyte antigens (C-KIT, DCT, PAX3, and TYR) in the epidermis and hair follicles

We tested normal control skin, NBUVB-treated skin, and untreated vitiligo skin. Our treated vitiligo skin included samples of perifollicular repigmentation pattern, collected at two time points (3 and 6 months). We compared the expression of study markers in the regions tested in four vitiligo patients treated with NBUVB for 3 months versus four vitiligo patients treated for 6 months and found similar expression patterns ($P > 0.05$, data not shown), suggesting that the repigmentation process was already complete by 3 months. As no significant variation in melanocyte markers expression was noted between the two time points, we included all eight samples in one group of "treated vitiligo" for the comparison with the untreated samples and with the normal control skin as well.

We compared all three groups (treated vitiligo, untreated vitiligo, and normal skin) in regard to markers expression using one-way analysis of variance. In the infundibulum, all markers exhibited significant variance of expression between treatment groups ($P < 0.05$; for DCT $P = 0.05$). In the bulge, markers expression did not vary significantly, except C-KIT ($P = 0.02$). In the bulb, there was no significant variance of marker expression between the three groups.

As shown in Figure 2 (which presents the results of multiple pairwise *post hoc* comparisons), the expression of melanocyte markers in both NBUVB-treated vitiligo skin and normal control skin was not significantly different (adjusted $P > 0.05$), being maximal in the epidermis, and gradually decreasing toward the bulge. In the untreated vitiligo skin, there were no melanocytes in the epidermis (Figure 2a, red bars absent), and there were very few in the infundibulum. NBUVB treatment was associated with increased expression of melanocyte markers in the epidermis (one sample *t*-test, $P < 0.001$), infundibulum (adjusted $P < 0.05$, except DCT for which $P = 0.05$), and bulge (adjusted $P > 0.05$), as compared with the untreated skin (Figure 2a–c and Supplementary Figure S1 online).

The expression of melanocyte markers was significantly higher ($P < 0.01$) in the treated epidermis, as compared with the treated bulge. Melanocyte marker expression in the normal skin and treated vitiligo skin was very similar (Figure 2a–c). The bulb of treated and untreated vitiligo and of normal skin showed a similar expression for melanocyte markers (Figure 2d).

In all three types of skin tested, TYR expression was absent in the bulge and lower infundibulum, and appeared only in the upper infundibulum (not shown), although our statistical analysis included samples along the entire infundibulum, between the epidermis and insertion of sebaceous gland duct.

Because the bulb melanocytes (derived from bulge melanocyte stem cells) were shown to be specialized in pigmentation of the hair shaft, and the bulge melanocytes to participate in the vitiligo repigmentation process (Myung and Ito, 2012), we focused our further experiments on the epidermis, hair follicle infundibulum, and hair follicle bulge, regions previously targeted in the mouse repigmentation models.

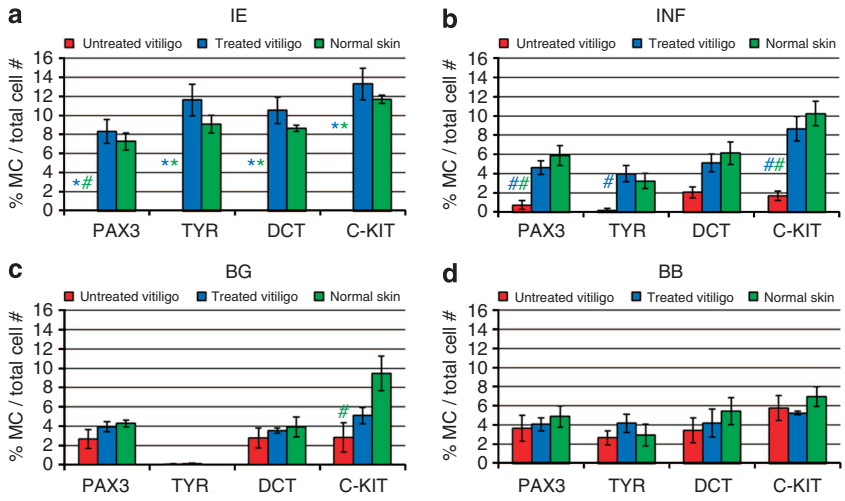

Figure 2. Graphical representation of melanocyte markers expression (PAX3, tyrosinase (TYR), dopachrome-tautomerase (DCT), and C-KIT). Marker expression was tested in interfollicular epidermis (IE) (**a**), hair follicle infundibulum (INF) (**b**), hair follicle bulge (BG) (**c**), and hair follicle bulb (BB) (**d**) of untreated vitiligo skin (red bars), narrow band UVB (NBUVB)-treated vitiligo skin (blue bars), and normal control skin (green bars). Significant differences in expression were observed for all markers in the epidermis (**a**) and some markers in the INF (**b**) of untreated vitiligo skin, as compared with NBUVB-treated or with normal skin. No significant differences were observed in the BG (**c**) and BB (**d**) of untreated vitiligo skin, as compared with NBUVB-treated vitiligo skin or with normal skin, except for C-KIT expression in the untreated BG compared with normal skin. ($P < 1.0E\text{-}03$ (*); $1.0E\text{-}03 \leqslant P \leqslant 5.0E\text{-}02$ ($^{\#}$); all other comparisons showed $P > 5.0E\text{-}02$ (not significant)). One-way analysis of variance with Tukey's multiple comparisons tests were used for statistical comparison. Each bar represents mean ± SEM ($n = 4$, untreated vitiligo patients; $n = 8$, NBUVB-treated vitiligo patients).

Distribution of DCT/TYR and C-KIT/TYR phenotypes in the NBUVB-treated epidermis and hair follicles

We examined the co-localization of C-KIT (present in immature melanocytes; Grichnik *et al.*, 1996) and DCT (marker of melanocyte lineage found from the bulge to the epidermis; Yamada *et al.*, 2013) with TYR (marker of differentiating melanocytes; Davids *et al.*, 2009). Consistent small immature populations of C-KIT+/TYR− were found in NBUVB-treated vitiligo skin in the epidermis, infundibulum, and bulge (Figure 3a–d). Another immature melanocyte phenotype (DCT+/TYR−) was maximal in the bulge, less in the infundibulum, and almost gone in the epidermis (Figure 3e–h). The expression of more differentiated phenotypes C-KIT+/TYR+ and DCT+/TYR+ was minimal in the bulge, more prevalent in the infundibulum, and reached the maximum in the epidermis.

Distribution of melanocyte stem cells and melanoblasts in the untreated and NBUVB-treated vitiligo and their proliferative potential

Figure 4a and c represent the melanocyte stem cells (the most immature population of melanocyte, carrying the phenotype C-KIT−/DCT+) and the melanoblasts (an immature population of melanocytes, carrying the phenotype C-KIT+/DCT+, which are more differentiated than the melanocyte stem cells; Yamada *et al.*, 2013). Both phenotypes were abundant in the untreated and treated infundibulum and bulge. In all regions tested of treated vitiligo, the melanoblasts were the majority population and also showed the highest proliferative capacity, as compared with the other C-KIT/DCT phenotypes. We also observed small subpopulations of proliferative melanocyte stem cells (C-KIT−/DCT+/KI-67+) in the treated and untreated bulge, and in untreated infundibulum, and proliferative melanoblasts (C-KIT+/DCT+/KI-67+) in the untreated bulge. The large percentage of melanocytes that were DCT+/C-KIT+ in the epidermis and infundibulum of treated vitiligo skin argue against all of these cells being melanoblasts. Interestingly, in the infundibulum of untreated skin, the subpopulation of proliferative melanoblasts was absent. The phenotype with unknown function C-KIT+/DCT− was similarly distributed along the hair follicle and the epidermis of treated skin, and higher expressed in all regions, as compared with untreated skin. Figure 4b illustrates some of the representative cell populations enumerated here.

In addition to the above data, our analysis of the normal control skin indicated a similar expression pattern for DCT, C-KIT, and KI-67 as compared with NBUVB-treated vitiligo skin (not shown).

Melanocyte migration and proliferation in the untreated and NBUVB-treated vitiligo

We next focused our analysis on the co-expression of DCT with MCAM (melanoma cell adhesion molecule, previously associated with melanocytic cell migration; Medic and Ziman, 2010; Medic *et al.*, 2011) and the proliferation marker KI-67 (Figure 5a and bi). In the NBUVB-treated vitiligo, we identified a non-proliferative and presumably migratory population (DCT+/MCAM+/KI-67−; Figure 5bi), which was similarly represented in the epidermis and infundibulum of treated skin and was nonsignificantly lower in the treated bulge. We also found a small proliferative and putatively mobile population DCT+/MCAM+/KI-67+

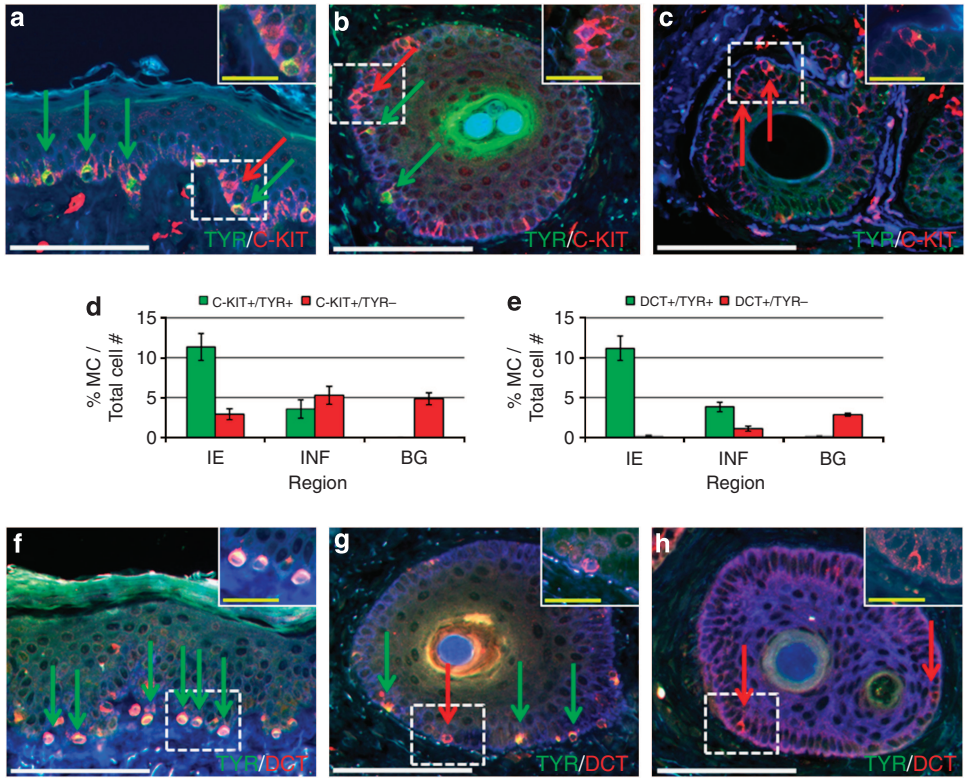

Figure 3. Distribution of C-KIT/tyrosinase (TYR) and dopachrome-tautomerase (DCT)/TYR populations in the interfollicular epidermis (IE) and hair follicles (HFs). Narrow band UVB (NBUVB)-treated skin sections, showing expression of melanocyte markers: anti-TYR (green) combined with either anti-C-KIT (red) or anti-DCT (red). Anti-K14 (blue) labeled basal keratinocytes. The precursor phenotypes—(C-KIT+/TYR −) and (DCT+/TYR −)—are shown by red arrows and the differentiated phenotypes—(C-KIT+/TYR+) and (DCT+/TYR+)—by green arrows in the IE (**a** and **f**), infundibulum (INF) (**b** and **g**), and bulge (BG) (**c** and **h**). Graphical representation in the regions tested of percent (C-KIT+/TYR −) precursors (red bars) and (C-KIT+/TYR+) differentiated cells (green bars) (**d**); percent (DCT+/TYR −) precursors (red bars) and (DCT+/TYR+) differentiated cells (green bars) (**e**). Each bar represents mean ± SEM ($n = 8$, NBUVB-treated vitiligo patients). White bars = 100 μm, yellow bars = 25 μm.

(Figure 5bi) in greatest proportion in the treated epidermis but also present in the treated infundibulum and bulge. In the treated skin, the majority of DCT+ melanocytes were non-migratory and non-proliferative (DCT+/MCAM − /KI-67 −). The migratory and proliferative phenotypes were similar in the bulge of treated (Figure 5bi) and untreated vitiligo (not shown). In the infundibulum of untreated vitiligo skin, the putatively migratory populations were absent (not shown).

In addition, MCAM expression in the DCT+ cells was significantly lower ($P \leqslant 0.05$) in the epidermis and hair follicles of normal control skin as compared with the treated vitiligo skin (not shown).

Melanocyte differentiation coupled with proliferation and migration in the untreated and NBUVB-treated skin

We examined the co-expression of TYR (marker of melanocyte differentiation) with MCAM and KI-67 in the infundibulum and the epidermis of NBUVB-treated vitiligo (Figure 5bii and c) and in untreated skin (not shown). We did not analyze the bulge because of the absence of TYR. We found two phenotypes, each expressed in more than 1/3 of the TYR+ cells: a differentiating, putatively immobile, and non-proliferative phenotype (TYR+/MCAM − /KI-67 − ; Figure 5bii), which was highly expressed in the treated skin, and a differentiating, putatively migratory, non-proliferative phenotype (TYR+/MCAM+/KI-67 − ; Figure 5bii). In addition, we identified a small differentiating, proliferative, and migratory population TYR+/MCAM+/KI-67+ (Figure 5bii) in the epidermis and infundibulum that represented <10% of total melanocytes. In contrast to the treated skin, the untreated infundibulum showed very few TYR-positive cells (Figure 2b), which did not express MCAM or KI-67.

Differential gene expression in the hair follicle bulge and epidermal melanocytes of the NBUVB-treated vitiligo

To see whether changes in the levels of melanocyte proteins C-KIT, DCT, PAX3, and TYR correlate with changes in gene expression, we examined the activation of genes encoding these proteins in three vitiligo patients treated 3 months with NBUVB (Supplementary Figure S2 online). We performed fluorescent laser capture microdissection of melanocytes located in the hair follicle bulge and the epidermis (panel a). Total RNA was extracted and then subjected to quantitative real-time reverse-transcriptase–PCR (qRT-PCR). For each individual sample, we observed a similar trend of remarkable upregulation in the epidermis as compared with the paired

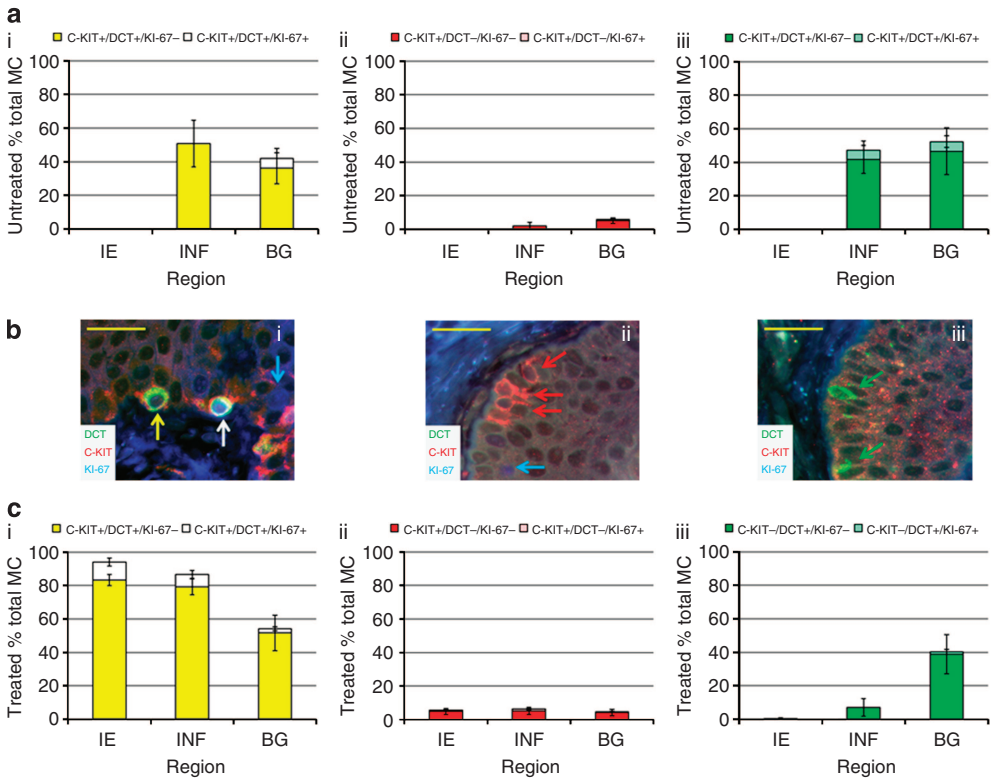

Figure 4. Distribution of melanocyte stem cell and melanoblast phenotypes and their proliferative capacity. (**a**) Untreated vitiligo: graphical representation of percent of melanoblast phenotype (C-KIT+/DCT+) (**ai**), C-KIT+/DCT− phenotype (**aii**), and melanocyte stem cell phenotype (C-KIT−/DCT+) (**aiii**) per total melanocytes (MC) in interfollicular epidermis (IE), infundibulum (INF), and bulge (BG). The most prevalent in INF and BG were non-proliferative melanoblasts (DCT+/C-KIT+/KI-67−; yellow bars, **ai**) and non-proliferative stem cells (DCT+/C-KIT−/KI-67−; green bars, **aiii**). Small subpopulation of proliferating melanoblasts DCT+/C-KIT+/KI-67+ are shown in the BG (white bar, **ai**). Small proliferative population of melanocyte stem cells (DCT+/C-KIT−/KI-67+) shown in the BG and IFN (light-green bars, **aiii**). (**b**) Narrow band UVB (NBUVB)-treated vitiligo: representative images of immunostaining in the IE and hair follicle, showing expression of anti-KI-67 (blue) combined with anti-C-KIT (red) and anti-DCT (green). Red arrows in the INF (**bii**) and red bars (**cii**) indicate cells that expressed only C-KIT (non-proliferating MC). The blue arrow shows cells that expressed only the KI-67 marker (likely keratinocytes) (**bi** and **bii**). Yellow Bar = 25 μm. (**c**) NBUVB-treated vitiligo: graphical representation of percent melanoblasts (C-KIT+/DCT+) (**ci**), C-KIT+/DCT− phenotype (**cii**), and the stem cells (DCT+/C-KIT−) (**ciii**) per total MC in IE, INF, and BG. The most prevalent phenotype observed in all three regions tested was non-proliferative (DCT+/C-KIT+/KI-67−) (**bi**, yellow arrow, and **ci**, yellow bars). A small population of proliferating MC DCT+/C-KIT+/KI-67+ decreased from the epidermal surface with the depth of the INF (**ci**, white bars, **bi**, white arrows). Each bar represents mean ± SEM ($n = 5$, untreated vitiligo patients; $n = 5$, NBUVB-treated vitiligo patients).

bulge sample (panel bi). When the results from the three samples were averaged (panel bii), there was a greater increase in *TYR* expression (~52-fold higher in the epidermis vs. bulge), compared with the increase in *KIT* (~10-fold), *DCT* (~14-fold), or *PAX3* (~3-fold) expression in the epidermis versus bulge. However, when all three samples were analyzed together, the higher expression in the IE compared with the bulge was significant for *DCT* ($P = 0.03$) and nonsignificant for *TYR* ($P = 0.05$), *PAX3* ($P = 0.08$), and *KIT* ($P = 0.16$).

NBUVB was associated with several melanocyte populations exhibiting proliferative, migratory, and differentiating abilities in vitiligo lesions

By combining the results of immunostaining experiments, we identified four main melanocyte populations in the UV-treated vitiligo skin, as presented in Figure 6 and in Supplementary Tables S3 and S4 online: a melanocyte stem cell population A (C-KIT−/DCT+/TYR−), a melanoblast population C (C-KIT+/DCT+TYR−), a population of differentiating cells D (C-KIT+/DCT+/TYR+), and a persistent population with unknown function B (C-KIT+/DCT−/TYR−). We identified the following: (i) immature populations of melanocytes A and melanoblasts C in the treated and untreated bulge, with small proportions of proliferative KI-67+ (A1 and C1) and/or putatively migratory MCAM+ (A2 and C2) subpopulations; the proportions of A and C populations increased nonsignificantly during NBUVB; (ii) a dominant immature melanoblast population C in the infundibulum of untreated skin, putatively immobile and non-proliferative. NBUVB treatment was associated with the expansion of the C phenotype and with the emergence of presumably mobile and proliferative subpopulations (C1, C2) in the treated infundibulum; (iii) very few differentiating melanocytes D in the untreated infundibulum, which lacked markers of proliferation and migration. A dominant, highly expanded phenotype D in the NBUVB-treated infundibulum, which contained subpopulations with markers of proliferation and migration

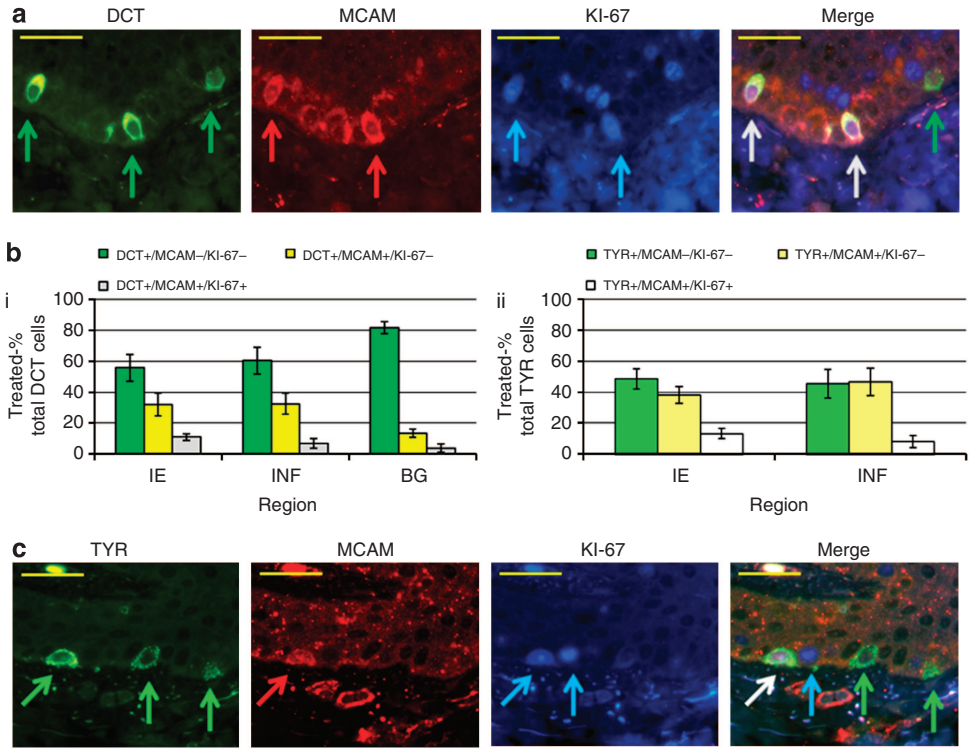

Figure 5. Assessment of putatively migratory (melanoma cell adhesion molecule (MCAM+)), differentiating (tyrosinase (TYR)+), and proliferative (KI-67+) phenotypes. (**a**) Representative immunostaining pictures of narrow band UVB (NBUVB)-treated vitiligo showing expression of markers in the interfollicular epidermis (IE): KI-67 (blue image, blue arrows) combined with MCAM (red image, red arrows) and dopachrome-tautomerase (DCT) (green image, green arrows). Triple-positive cells (DCT+/MCAM+/KI-67+) are presented in the merged image (gray arrows), as well as one cell DCT+ only (green arrow). Yellow bar = 25 μm. (**b**) Graphical representation of the percent presumably migrating (MCAM+) and non-migrating (MCAM –) melanocytes co-expressing DCT and KI-67 (**bi**) or TYR and KI-67 (**bii**) per total melanocytes in IE, infundibulum (INF), and bulge (BG) of NBUVB-treated vitiligo skin. **bi** shows a large population of putatively non-migratory and non-proliferative DCT+/MCAM – /KI-67 – cells in all three regions (green bars), an increasing population of presumably migrating but non-proliferating melanocytes (DCT+/MCAM+/KI-67 –) from the BG to the IE (yellow bars), and a small population of putatively migrating proliferative melanocytes (DCT+/MCAM+/Ki-67+) highest in the IE (gray bars). **bii** shows similar trends for TYR. The BG was not analyzed for TYR as the melanocytes were TYR – . Each bar represents mean ± SEM. (**c**): Representative immunostaining images of NBUVB-treated vitiligo showing expression of markers in the INF: anti-KI-67 (blue image, blue arrows) combined with anti-MCAM (red image, red arrows) and anti-TYR (green image, green arrows). A single triple-positive cell is seen in the merged image (white arrow), as well as two TYR+ cells (green arrows) and a KI-67+ keratinocyte (blue arrow). Each bar represents mean ± SEM ($n = 5$, NBUVB-treated vitiligo patients). Yellow bar = 25 μm.

(D1, D2); (iv) a phenotype C-KIT+/DCT+, also TYR+ (D), present in almost all melanocytes emerged during NBUVB in the depigmented epidermis, containing putatively mobile proliferative or non-proliferative subpopulations (D1, D2) but also a dominant differentiating, non-migratory and non-proliferative subpopulation C-KIT+/TYR+/DCT+/MCAM – /KI-67 – . About 10% of the differentiated melanocytes in the epidermis and infundibulum were KI-67+; (v) a small population B of C-KIT+/DCT – phenotype, in all regions with melanocytes, which represented about 5% of the total in the NBUVB-treated skin and contained small subpopulations of KI-67+ or MCAM+ cells (Figure 4 and Supplementary Table S4 online).

DISCUSSION

In this study, we tried to provide a better understanding of the repigmentation process in vitiligo. Our work studied the perifollicular pattern of repigmentation, because this was the most commonly observed in our clinic. The predominance of this pattern claimed by us and others (Parsad *et al.*, 2004; Yang *et al.*, 2010) suggests that the hair follicle is the main reservoir that supplies melanocytes in repigmenting human vitiligo. Other patterns, like marginal and diffuse repigmentation (rarely observed by us and less often than the perifollicular pattern by others (Yang *et al.*, 2010)), suggest the presence of a secondary reservoir, possibly derived from the more immature basal melanocytes located at the lesional borders.

Our immunostaining study showed that the hair follicle bulge of treated and untreated vitiligo and the untreated infundibulum contained only amelanotic melanocytes (i.e., they expressed the melanocyte markers DCT and/or C-KIT and/or PAX3, but they were TYR – and Fontana-Masson-; Figures 2,3,4 and Supplementary Figures S1 and S3 online). Fontana-Masson+ cells and TYR+ cells were seen only in the upper infundibulum and the epidermis of treated skin (Supplementary Figures S1 and S3 online). NBUVB treatment was associated with significantly increased expression of melanocyte markers in the vitiligo spots, the most striking

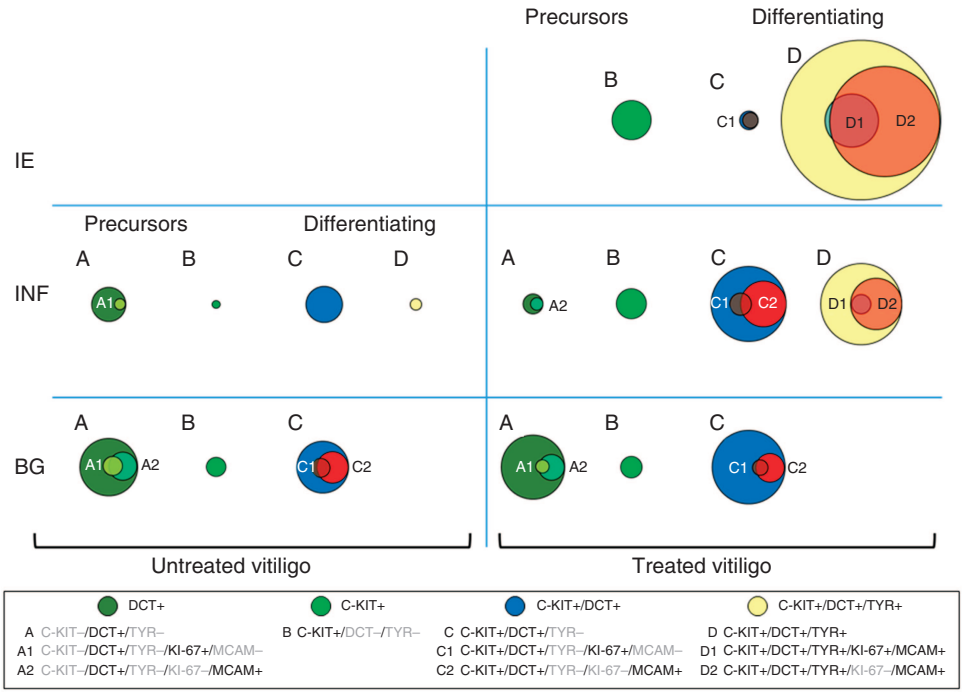

Figure 6. Diagram of melanocyte precursors and more differentiated phenotypes in treated and untreated vitiligo. The precursors and more differentiated phenotypes are represented with different colors in the bulge (BG), infundibulum (INF), and interfollicular epidermis (IE). The relative diameter of each circle in each study region represents the estimated percent of melanocytes exhibiting particular phenotypes in each study region, normalized to the average number of melanocytes in each region (melanocyte percentages presented in the Figure 2 and Supplementary Table S1 online). Percentages of each phenotype and circle diameters are summarized in the Supplementary Tables S3 and S4 online.

contrast being observed between the untreated depigmented epidermis (devoid of melanocytes) and the treated pigmented epidermis, which was heavily DCT+, C-KIT+, PAX3+, TYR+, and strongly Fontana-Masson+ (Supplementary Figures S1 and S3 online). The increase in melanocyte marker expression was reflected by an increase in cell numbers, and also in staining intensity, which was high in the treated epidermis and weaker in the treated and untreated hair follicle bulge (Supplementary Figure S1 and S3 online). In the treated epidermis, the markers expression was significantly elevated as compared with the treated bulge, and this result was paralleled at the transcription level by our qRT-PCR analysis (Supplementary Figure S2b online). The results of our gene expression analysis are consistent with the function of these pigmentation genes. Both *DCT* and *TYR*, genes coding for proteins that control melanogenesis, were highly increased in the epidermis of NBUVB-treated vitiligo skin, reflecting high production of melanin here. *KIT*, with attributed roles in melanocyte proliferation and migration, was also higher expressed in the epidermis (compared with the bulge). *PAX3* showed slight increase in expression in the epidermis compared with the bulge, consistent to the fact that it is a transcription factor gene with rapid fluctuations and which can alternatively activate and downregulate downstream pigmentation genes (Kubic *et al.*, 2008). Our observation of the low expression of pigmentation genes in the bulge verified previous studies (Nishimura *et al.*, 2010), attributing the inhibitory role of gene transcription to TGF-β signaling, which maintains melanocyte stem cell immaturity and quiescence in the bulge.

We found no difference in melanocyte marker expression between NBUVB-treated and normal skin (Figure 2 and Supplementary Table S1 online). This observation indicates that NBUVB exposure for 3–6 months can bring the depigmented skin to an apparently normal status.

Our human vitiligo model revealed a similar distribution of "melanoblast" (C-KIT+/ DCT+) and "melanocyte stem cell" (C-KIT−/DCT+) phenotypes (Figure 4), as was previously reported in mice (Yamada *et al.*, 2013). Like Yamada, we assume that the (C-KIT−/DCT+) phenotype labeled melanocyte stem cells because of its almost exclusive location in the bulge and because DCT is a lineage marker expressed in melanocyte precursors earlier than C-KIT (Nishimura *et al.*, 2002). However, in our model, the C-KIT+/DCT+ population included a broader stage than the immature "melanoblast" that was earlier described by Yamada *et al.*, 2013.

In our study, melanocyte proliferation after NBUVB was indirectly reflected by the observation of increased melanocyte numbers in all regions tested (Figure 2) and was directly quantified by KI-67 co-expression with C-KIT, DCT, and TYR (Figures 4 and 5). Melanocyte proliferation was nonsignificantly higher in the bulge of treated skin (as compared with the untreated skin) and was significantly higher in the infundibulum and the epidermis. These results are in accord with our recent studies on UV-induced DNA damage, showing that single-dose NBUVB irradiation on human skin penetrates the hair follicle and induces cellular changes in

the bulge, as well as in more superficial regions of the skin (White *et al.*, 2013). Two recent studies on transgenic mice (Chou *et al.*, 2013; Yamada *et al.*, 2013) also reported melanoblast or melanocyte proliferation in the hair follicle and/or the epidermis induced by stimuli like UVB or wounds created in the mouse skin.

Although we have not yet been able to follow melanocyte migration in real time, we evaluated the putatively migratory capacity more indirectly through immunostaining (Figure 5). We identified a presumed migratory population of melanocytes (DCT+/MCAM+) that was minimally expressed in the bulge and of which expression increased in the infundibulum and the epidermis. Two studies (Nishimura *et al.*, 2002; Chou *et al.*, 2013) utilized direct labeling of melanocytes (carrying the DCT-LacZ+ mutation) to demonstrate the melanocyte migration from the bulge to the epidermis induced by different stimuli (like forced Slf expression in epidermal keratinocytes carrying the Slf-K14 transgene, UVB exposure, or wounds created on the mouse skin). More recently, the results of an immunostaining study of human vitiligo skin (Kovacs *et al.*, 2015) suggested the migratory capacity of melanocytes (horizontal migration to depigmented areas) in response to punch grafting followed by treatment with Khellin+UV light.

In our model, the melanocyte precursors showed differentiation abilities in the upper infundibulum by gradually exhibiting TYR expression, a process that paralleled proliferation and migration and that continued in the epidermis (population TYR+/MCAM+/KI-67+; Figure 5b and c). A similar robust melanocyte maturation process (induced by different stimuli, like UVB exposure, or by forced expression of Slf in the epidermis) was described in the hair follicle melanoblasts and epidermal melanocytes of different transgenic mouse models (Nishimura *et al.*, 2002; Chou *et al.*, 2013; Yamada *et al.*, 2013).

Our study suggests a possible association of the following observations (also represented in the Figure 6) with NBUVB treatment: (i) a nonsignificant increase in the expression of precursor populations in the bulge; (ii) a noticeable increase in the expression of the melanocyte differentiating phenotype D in infundibulum, the emergence of phenotype D in epidermis, as well as the emergence of proliferative and migratory differentiating subpopulations D1 and D2 in both infundibulum and the epidermis; (iii) the emergence of subpopulations of putatively mobile and proliferative melanoblasts (C1, C2) in the infundibulum; (iv) a gradual decrease in melanocyte precursors from the bulge to the epidermis, which suggests a possible role of NBUVB in maturation of epidermal melanocytes; (v) the absence of the classical stem cell population A (C-KIT−/DCT+/TYR−) as the only putative precursor population identified in the epidermis, which might represent a secondary melanocyte germ there and in the upper infundibulum. As we discussed above, the hypothesis of an intra-epidermal reservoir of melanocytes has been previously claimed based on clinical (Yang *et al.*, 2010) but also experimental observations (Grichnik *et al.*, 1996; Davids *et al.*, 2009), although clarification of this aspect is awaited through future experiments.

In sum, the presence of small subsets of KI-67+ and/or MCAM+ cells of melanocyte lineage (DCT+) in the melanocyte stem cell, melanoblast, and differentiated melanocyte populations supports the concept that proliferation and migration within the bulge, infundibulum, and the epidermis are essential for the development of the large population of TYR+ melanocytes in the epidermis of NBUVB-treated vitiligo. A significant subpopulation of TYR-proliferating melanoblasts is not found in the treated epidermis, supporting the view that the proliferating cells that repopulate depigmented vitiligo epidermis arise in a TYR+ population in the upper infundibulum and the epidermis. We propose that NBUVB treatment is associated with a stepwise proliferation and migration from the bulge to the epidermis: proliferation and migration of melanocyte stem cells, then melanoblasts, and then differentiating TYR+ melanocytes. The upper infundibulum is a key site of melanoblast differentiation to TYR+ cells; these cells then continue to proliferate and migrate to the epidermis. Once they reach the epidermis, they can continue to proliferate, migrate, and, in addition, to terminally differentiate, as part of the major epidermal melanocyte population.

Our human model and previous animal models support the concept that the hair follicle bulge is the location of melanocyte stem cells that are the major precursors for the melanocyte repopulation of the epidermis.

MATERIALS AND METHODS

Sample collection

Skin biopsies were performed at the Dermatology Clinic at University of Colorado Hospital. Written informed consent was obtained from all subjects, and the study was approved by Human Subjects Committee (COMIRB) at the University of Colorado.

Mapping the key regions of the hair follicle

Both paraffin and frozen transversal sections were immunostained for the hair follicle bulge and infundibulum mapping with anti-CD200, anti-K15, and anti-Desmin antibodies using every 10th slide according to established methods.

Antibodies and immunohistochemistry

The primary and secondary antibodies used are listed in the Supplementary Materials and Methods online, and standard procedures were used for immunohistochemistry.

Statistical analysis

For each sample, quantification of cells positive for a given marker was done, and the differences in percentages for each site/marker were analyzed, as presented in the Supplementary Materials and Methods online.

To build the Venn diagram (Figure 6), we used the raw data generated by our 2- and 3-marker immunostaining assays (Supplementary Table S5 online) and a fractional factorial design (Box *et al.*, 2005; Collins *et al.*, 2009) to impute summary statistics of the 2-, 3-, 4-, and 5-marker combinations that were not possible to measure directly.

Fluorescent laser capture microdissection

We captured 100 individual melanocytes from the epidermis and hair follicle bulge using frozen sections of three NBUVB-treated

vitiligo patients. Sections were rapidly immunostained using a mouse monoclonal antibody to NKI-beteb (Horikawa *et al*. 1996).

RNA extraction and qRT-PCR

Total RNA was extracted from the laser captured melanocytes and then amplified, as described in the Supplementary Materials and Methods online. qRT-PCR was performed with primers designed by ourselves and standard procedures. Primer pairs are shown in Supplementary Table S2 online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the University of Colorado Anschutz Medical Campus Skin Disease Research Center (P30AR057212), American Skin Association and by UC Denver LCM Shared Resource funded by the Cancer Support Grant NCI P30CA046934. We are grateful to our team in Dermatology Clinic at UCH for sample collection: Nina Rukavina, Barnard Howard, Fernando Belleza, Ellen Barry, Katherine Wilson, Colleen Powell, Irish Espejo, Ayesha Gonzales, and Tracey Seckman. We are grateful to Smaranda Birlea (for cell counting), Dr Vince Hearing (for the generous DCT antibody gift), and Drs Manabu Ohyama, Randall Cohrs, Mark Burgoon, and Caroline Lambert for valuable advice. We thank the University of Colorado Skin Cancer Biorepository and National Disease Research Institute for providing human skin samples.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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